MODIFIED EXOSOMES AND USES

The present invention relates to the areas of biology and immunology. It relates to membrane vesicles containing molecules, especially antigenic molecules, of predetermined structure, and their uses. It relates more particularly to vesicles containing recombinant molecules of the major Histocompatibility complex, and their use as immunogenic agents or as diagnostic or therapeutic tools. The invention also concerns methods for producing these vesicles, genetic constructs, cells and compositions that can be used to implement the methods of the invention.

The specificity of antigen recognition is a major characteristic of immunity system cells. B-lymphocytes recognize antigens in native form. T-lymphocytes recognize the complexes formed by the association of peptides derived from antigen degradation with molecules of the Major Histocompatibility Complex (MHC). The peptides derived from antigens synthesized by organism cells (tumoral or viral antigens) combine with class I molecules of the MHC which are recognized by cytotoxic T-lymphocytes. The peptides derived from exogenous antigens combine with class II molecules of the MHC which are recognized by the auxiliary T- lymphocytes. The identification of the peptides presented by MHC molecules and recognized by the cytotoxic (CD8) or auxiliary (CD4) T-lymphocytes was the start of new therapeutic and vaccine

strategies. This progress in immunotherapy requires the development of techniques to evaluate antigen specific immunity response.

Antigenic peptides combine with the MHC molecules in intracellular compartments. For class II molecules, these are made up of vesicles contained in a larger granule belonging to the endocytic route (Peters et al., Nature 349 (1991) 669). Their fusion with the plasma membrane leads firstly to the expression of peptide-MHC complexes on the cell surface, and secondly to the secretion of these vesicles called exosomes.

Research by Raposo et al. (J.Exp.Med.183 (1996) 1161) has shown that B-lymphocytes are able to secrete exosome vesicles carrying class II molecules of the MHC. Also, Zitvogel et al. (Nature medicine 4 (1998) 594) have demonstrated the production of particular membrane vesicles by the dendritic cells (called dexosomes) having advantageous properties. Therefore, these vesicles express class I and class II molecules of MHC and are able, after sensitization to the corresponding antigens, to stimulate the *in vivo* production of cytotoxic T-lymphocytes and to bring about whole or partial resorption of tumours.

The present invention concerns new methods and compositions that can be used in the areas of biology and immunology. More particularly, the present invention describes new membrane vesicles whose composition has been modified in determined manner. In particular, the present invention describes a new

method enabling the tailored production of vesicles expressing molecules of the MHC complex of known composition, optionally complexed with antigenic peptides of determined structure. With the present invention, it is therefore possible to modify the composition of membrane vesicles in controlled manner, and consequently to create products that are particularly advantageous at therapeutic, diagnostic or even experimental level.

The vesicles described up until now, at the very best, contain endogenous MHC molecules, that is to say MHC molecules expressed by the cell from which they are derived. On this account, these molecules are of varied structure, not always identified, and are generally multiple, depending upon the HLA type of the organism from which they are produced. On the contrary, with the present invention it is possible to produce membrane vesicles carrying MHC molecules of defined composition. Also, the vesicles of the invention have the advantage of containing a high number of MHC molecules determined in this manner, and they provide powerful immunogenic properties.

The present invention particularly concerns membrane vesicles containing molecules of pre-determined structure, especially MHC molecules of pre-determined structure. The present invention relates in particular to membrane vesicles containing MHC-peptide complexes of predetermined structure. The present invention also concerns a method for modifying the

composition of a membrane vesicle comprising the insertion, into a cell producing said vesicle, of a nucleic acid containing a hybrid region made up of a coding region fused to an addressing region, or a nucleic acid coding for a protein or polypeptide which, alone or associated with one or more proteins, is naturally addressed into these membrane vesicles.

The present invention also concerns membrane vesicles comprising determined antigenic molecules anchored in the membrane part. Said molecules may be exposed outside the vesicles or, on the contrary, enclosed in the cytosolic fraction. The present invention further concerns membrane vesicles containing molecules of pre-determined structure, exposed on their surface, enabling their purification in particular using affinity methods. The present invention also concerns membrane vesicles such as defined above also comprising a tracer. With said tracer it is possible in particular to detect vesicles in a sample, for example for their *in vivo* follow-up.

The invention also concerns a method for preparing the above-defined vesicles and the use of these vesicles. For example, these vesicles may be used as immunogenic agents for the preparation of antibodies. In particularly advantageous manner, these vesicles are used to produce antibodies restricted to the MHC, that is to say specific to a peptide-MHC molecule complex.

One first objective of the invention is more particularly a membrane vesicle, characterized in that it contains a recombinant molecule of the Major Histocompatibility Complex.

The term membrane vesicle, in the meaning of the invention, particularly denotes any vesicle made up of a lipid bi-layer enclosing a cytosolic fraction. These vesicles are generally produced by launching from cells, and are therefore also called "exosomes" in the present application. The membrane vesicles (or exosomes) of the invention generally have a diameter of approximately 60 to 80 nm. Also, these vesicles advantageously carry membrane proteins which have the same orientation as in the plasma membrane of the cells from which they are derived.

The present invention will demonstrate below that it is possible to modify the composition of exosomes in controlled, specific manner. More particularly, the present invention shows that it is possible to produce membrane vesicles expressing recombinant molecular complexes of (pre)determined composition. As will be illustrated later on in the disclosure, said vesicles have particularly advantageous properties both from a therapeutic viewpoint and from a diagnostic and experimental viewpoint.

The present invention arises firstly from the selection of special cell populations for the production of membrane vesicles. The present invention

also arises through the finding that it is possible to insert recombinant molecules in these cells, by genetic route, and that these recombinant molecules are subsequently expressed in dense, functional manner in the exosomes .

One of the first elements of the invention therefore lies in the definition and identification of the cell population used for the production of membrane vesicles. Advantageously, the cell used is a cell containing internal secretion vesicles, a cell that can be cultured, genetically modified and whose internal vesicles can preferably be secreted under the effect of outside stimulation. This mainly concerns mammalian cells, in particular animal cells, but also cells of human origin. Also primary cultures may be used or immortalized lines.

In particularly advantageous manner, the initial cells are essentially free of MHC molecules, that is to say they express no or only a few endogenous MHC molecules. This characteristic can prove to have great importance in some applications, as will be illustrated below.

Different types of exosome-producing cells have been described in the literature, such as for example dendritic cells or B-lymphocytes. Nonetheless, these cells are generally difficult to transfect and contain a high number of endogenous MHC cells. On this account, although they may be used to implement the invention, the vesicles of the present invention are more

preferably able to be obtained from mastocyte cells or mastocyte-derived cells.

Under one particular embodiment, the membrane vesicles of the invention are preferably prepared from mastocyte or mastocyte-derived cells.

Mastocytes group together a set of cell types derived from medullary precursors lying, after differentiation, in epithelia such as the skin, lung, intestines or spleen (Smith and Weis, Immunology Today 17 (1996) 60). These cells are characterised essentially in that their cytoplasm is for the most part made up of granules which contain histamine, as well as heparin or proteases, and in that they express receptors on their surface which have a strong affinity for E immunoglobulins (IgE). Also, a further advantage of the use of mastocytes according to the invention lies in the possibility of initiating (in particular of strongly stimulating) exocytosis (i.e. the release) of exosomes by different treatments. Therefore, it is possible to regulate vesicle production by treatment in the presence of a calcic ionophor or, in more physiological manner, by the stimulation of receptors having a strong affinity for IgEs .

These cells offer properties of particular interest for the implementation of the present invention, namely the presence of internal secretion vesicles, their possible culture and the induction of massive exocytosis. Also, it has now been shown as described in the examples, that these cells can also be

genetically modified in stable manner, which provides a particularly advantageous property for the implementation of the present invention.

More specifically, the vesicles of the invention have a diameter of approximately 60 to 80 nm and are produced from mastocyte or mastocyte-derived cells.

In one preferred embodiment, the membrane vesicles of the invention are essentially free of endogenous MHC molecules. The absence of endogenous MHC molecules (that is to say MHC molecules from the vesicle-producing cell) can be evidenced with specific antibodies using conventional techniques. It can also be evidenced by the selectivity of the antibodies obtained by immunization with the vesicles. As indicated in the examples, the vesicles of the invention are, in one particularly advantageous embodiment, able to induce in animal the production of specific antibodies for the defined recombinant molecules they express, without detecting antibodies directed against non-genetically modified cells. The term "essentially" free means that some MHC molecules may be present in very low quantities that are difficult to detect by conventional methods and have no notable impact on the antigenic specificity of the vesicles of the invention.

Particular membrane vesicles of the invention are more specifically characterised through the following properties :

- they are essentially free of endogenous MHC molecules

- they carry one or more recombinant molecules of defined structure, for example recombinant peptide-MHC complexes, of defined composition.

Said vesicles of the invention are advantageously produced from cells derived from mastocytes which are essentially free of endogenous MHC molecules. In this respect, it is known that mastocytes accumulate class II MHC molecules in their secretion granules. In particular, mastocytes are able, in preferential manner, to accumulate MHC-II-peptide complexes in special multivesicle intracellular compartments, the secretion granules (Raposo et al., Mol. Biol. Cell 8 (1997) 2619). These cells, taken from a mammal, therefore contain endogenous MHC molecules. In particularly advantageous manner, the cell lines used in this invention are derived from mastocytes that are essentially free of endogenous MHC molecules. Different mastocyte cell lines have been described in the literature. The present invention will demonstrate below that some of these lines have low levels of MHC molecules, and are therefore particularly advantageous for the implementation of the invention. By way of illustration, mention may be made of lines derived from RBL cells (Rat Basophilic Leukemia) filed with ATCC under number CRL1378 (Kulczycki et al., J. Exp. Med. 139 (1974) 600), the KU-812 line (Butterfield et al., Leukemia Res. 12 198) 345), or even immature human

mastocyte cell lines such as the HMC line (Nilsson et al., Scand. J. Immunol. 39 (1994) 489). A particular line example is the RBL-2H3 line (Barsumian et al., Eur. J. Immunol. (11 (1981) 317). Evidently, any other cell having the properties described above may be used.

For the present invention the expression "defined composition" denotes in particular the fact that the vesicles of the invention have for example considerable antigenic and haplotype specificity. The vesicles described in the prior art generally express MHC molecules of various, unknown haplotypes. On the contrary, the preferred vesicles of the invention express recombinant molecules whose haplotype is predetermined in precise manner. The term "recombinant" indicates that the molecule results from the expression, in the vesicle-producing cell, of a recombinant nucleic acid coding for this molecule. The membrane vesicles of the present invention are therefore more preferably produced from cells, in lines, which are genetically modified to express constituents of predetermined structure.

As indicated above, the vesicles of the invention advantageously express defined molecules of the MHC.

The molecules of the human MHC are grouped under two distinct classes, class I MHC molecules and class II MHC molecules.

In one particular embodiment, the vesicles of the invention express one or more recombinant class II

molecules of the major Histocompatibility complex. In this respect, class II molecules of the human MHC are made up of two chains, an α chain and a β chain, the β chain conferring allelic specificity to the complex.

Under a specific variant, the vesicles of the invention more particularly express a recombinant α chain of a class II molecule of the major Histocompatibility complex. Under another specific variant, the vesicles of the invention more particularly express a recombinant α chain and a recombinant β chain of a class II molecule of the major Histocompatibility complex.

Different types of human MHC II molecules have been identified, characterised and sequenced (see for example Immunogenetics 36 (1992) 135). Preferential mention may be made of molecules of type DR1 to DR13, in particular DR1, DR2, DR3, DR4, DR5, DR6 and DR7. The DNA coding for human DRs, in particular DR1 to 13, may be easily isolated from cells, banks or plasmids using conventional molecular biology techniques. These sequences have been described in particular in Bodmer et al., (Tissue antigens 44 (1994) 1). Preferably, the exosomes of the invention therefore express a class II molecule of the MHC comprising an α chain, and a β chain chosen from among haplotypes DR1 to DR13, or further preferably from DR1 to DR7.

In one specific example, the invention concerns any membrane vesicle comprising a recombinant α and/or β chain of a MHC-II molecule of the DR1 haplotype.

In another embodiment, the vesicles of the invention express one or more class I recombinant molecules of the Major Histocompatibility Complex. The class I MHC molecules are also made up of two chains, the transmembrane and polymorphic α chain, and β2-microglobulin, which is constant and soluble. In man, three genetic loci encode the α chain, designated A, B and C. In conventional MHC-I molecules, each locus A, B and C of the α chain is subject to allelic variation. Hence, the alleles are denoted A1, A2 A3 etc., A10, B1, B7, B37, B54 etc., CW3, CW6 etc. (see for example Bodmer et al., cited above and Immunogenetics 36, 1992, also cited above).

Preferably, the exosomes of the invention express an α chain of a conventional MHC-I molecule, that is transmembrane and polymorphic. Further preferably, it is an α chain of a MHC-I molecule of allele A1, A2 or A3.

In one particular embodiment, the exosomes of the invention express an α chain of a non-conventional MHC-I molecule, that is to say non-polymorphic. Unlike so-called "conventional" MHC-I molecules, subject to substantial polymorphism, there exists in man "non-conventional" MHC-I molecules which are essentially non-polymorphic. Such molecules have for example been

described in Bendelac et al. (Ann. Rev. Immunol.(1997) 535). One preferred example of a non-conventional MHC-I according to the invention is the molecule Cd 1.

Evidently, any other molecule of the human MHC-I may be expressed within the scope of the present invention.

In one specific example, the invention therefore concerns any membrane vesicle containing a recombinant protein of a MHC-I molecule.

In one particular variant, the vesicles of the invention comprise several class I and/or class II MHC molecules. One advantageous vesicle for example comprises 2 MHC-II molecules of different haplotypes, or more. Any other combination of MHC molecules is evidently possible, such as for example MHC-I and MHC-II.

The vesicles of the invention expressing one or more defined MHC complexes are particularly advantageous since they enable the presentation of a given antigenic peptide in a defined MHC context. In this respect, in one more preferred embodiment of the invention, the membrane vesicles contain a complex between a defined peptide and the recombinant molecule of the Major Histocompatibility Complex.

The vesicles of the invention may also comprise one or more other, heterologous, molecules of interest, in addition to or instead of the above-mentioned MHC molecules. In this respect, in one particular variant,

the invention concerns membrane vesicles produced from mastocyte or mastocyte-derived cells, characterised in that they comprise one or more heterologous molecules of interest. The term "derived" mastocyte cell designates transformed and/or immortalised lines and/or obtained from cells of mastocytes or basophils and having the properties of mastocyte cells (accumulation of internal secretion vesicles). The term "heterologous" indicates that the molecule of interest is not present, in this form, in the exosomes of the invention in their natural state.

The molecules of interest carried by or contained in the exosomes of the invention may be any protein, polypeptide, peptide, nucleic acid, lipid, and any substance of interest (of chemical, biological or synthetic nature). These molecules may be of recombinant nature and may be inserted in the producing cell or directly in/on the exosomes. More particularly preferred types of molecules of interest are especially MHC molecules, antigens (whole or in peptide form), receptor ligands, (specific) receptors of ligands, nucleic acids, pharmacological products, tracers or even peptides or proteins enabling vesicle purification.

As antigen, special mention may be made of any protein, in particular a cytoplasmic protein or of viral or tumoral origin. As preferred examples of proteins of viral origin, any cytoplasmic or membrane protein may be cited expressed by the EBV, CMV, HIV

viruses, measles, hepatitis etc. These are more preferably cytoplasmic proteins, that is to say essentially invisible to the immunity system in the conventional infection process, and therefore little immunogenic under natural conditions, or they may also be membrane proteins or protein fragments. As preferred examples of proteins of tumoral origin, particular mention may be made of the p53 proteins (wild or any mutated form present in the tumour), MAGE (in particular MAGE 1, MAGE 2, MAGE 3, MAGE 4, MAGE 5 and MAGE 6), MART (in particular MART 1), Gp100, the ras proteins (wild or mutated p21), etc. Evidently any other protein of interest can be expressed in or on the surface of the exosomes of the invention, using the teaching of the present application.

In this respect, the recombinant antigenic molecules may be present either on the surface of the vesicles (exposed), or inside the vesicles. Indeed, in particularly surprising manner the inventors found that vesicles of the invention containing, in their cytosol, a recombinant antigen (p53 in particular) were able to induce, in animal, a very high production of antibodies directed against this antigen.

Among the ligand receptors, mention may be made in general of any ligand receptor either natural or derived from genetic manipulation. In particular, they may be any hormone, growth factor, lymphokine, trophic factor, antigen receptor, etc. Special mention may be made of the receptors of interleukins IL1 to IL15, the

growth hormone receptor, or the receptor of stimulation factors of colonies of granulocytes and/or macrophages (G-CSF, GM-CSF, CSF, etc). One particular example of ligand receptor is made up of a single chain antibody (ScFv) which enables interaction with a specific ligand. Another particularly advantageous example in the meaning of the invention is represented by the T-lymphocyte antigen receptor (TcR). Exosomes of the invention expressing on their surface one or more defined TcRs form particularly advantageous analysis and diagnostic tools as will be seen in detail later on.

As pharmaceutical product, mention may be made of any active substance, of a chemical nature, such as for example pharmaceutical products prepared using conventional chemistry techniques. Any protein, polypeptide or peptide having biological activity may also be cited such as for example a toxin, hormone, cytokine, growth factor, enzyme, tumour suppresser, etc.

The nucleic acid may be any DNA or RNA coding for a protein, polypeptide or pharmacological peptide such as mentioned above, and any other nucleic acid having a particular property (antisense, antigen, promoter, repressor, binding site for a transcription factor, etc.). It may be an oligonucleotide, a coding phase, an artificial chromosome, etc.

The vesicles of the invention carrying a ligand receptor may be used to detect any interaction of

ligand-receptor type, in particular of low affinity, in any biological sample, as will be explained in greater detail in the remainder of this disclosure. Also, such vesicles may also be used to transport substances of interest (protein, peptide, nucleic acid, chemical substance, etc.) towards cells. Therefore, the exosomes of the invention may be used, generally, for the transport and transfer of any molecule into cells, *in vitro*, *ex vivo* or *in vivo*. The invention therefore concerns any vesicle such as described above comprising a heterologous molecule of interest, that can be used as a transfer vector for said molecule into a cell.

In a more preferred embodiment, the exosomes of the invention are used for the oriented transfer of substances of interest towards selected cell populations. Consequently, it is possible to prepare vesicles of the invention comprising a substance of interest (toxin, hormone, cytokine, recombinant nucleic acid, etc.)and expressing on its surface a ligand receptor or a receptor ligand, and to place said vesicles in contact with cells expressing the corresponding ligand or receptor. With this approach, it is therefore possible to achieve targeted, efficient transfer.

In this respect, one particular object of the invention lies in a vesicle such as defined above, characterised in that it expresses a ligand receptor and in that it comprises a heterologous molecule of interest.

The vesicles of the invention may also contain a peptide or a recombinant protein enabling vesicle purification. Consequently, the invention in effect describes the possibility of genetically modifying the composition of exosomes, and hence of causing them to express a special "label" molecule, enabling its purification. In particular, it is possible to obtain an exosome exposing a peptide of particular structure, which may be easily detected and captured by a receptor molecule. In one particular example, an exosome is produced comprising, in its structure, a peptide molecule comprising the His6 pattern (i.e. 6 consecutive histidine residues). The presence of such residue on the surface of exosomes makes their purification easy on a support medium functionalised with nickel. Other recombinant peptides of this type may be used, as for example the c-myc tag, VSV or HA.

Finally, in one particular variant, the vesicles of the invention also contain a tracer. The tracer may be of different nature (enzymatic, fluorescent, radioactive, etc) and present in the vesicle or on its surface. One preferred labelling is non-radioactive, such as for example fluorescent labelling. More preferably, the tracer used is a fluorochrome or an enzyme with a chromogenic substrate. Labelling may be made directly on the producer cell, or on the exosomes produced.

The invention also concerns any composition comprising one or more membrane vesicles such as

defined above. The compositions of the invention may also comprise a plurality of membrane vesicles such as defined above carrying different recombinant molecules. In particular, a composition of the invention may comprise membrane vesicles such as defined above, carrying recombinant molecules of the MHC of different haplotypes in association with one same antigenic peptide. The compositions may also be compositions comprising membrane vesicles as defined above carrying recombinant MHC molecules of one same halotype, associated with different antigenic peptides for example. Other combinations of vesicles of the invention are evidently possible.

The compositions of the invention generally comprise a vehicle such as a buffer, saline or physiological solution etc. enabling the structure of the vesicles to be preserved. They may also comprise any stabilising, surfactant agent etc. preferably compatible with biological use (*in vitro* or *in vivo*). These compositions may be stored in any appropriate device such as a tube, bottle, ampoule, flask, pouch etc. and stored at 4°C or at -20°C for example. Typical compositions according to the invention comprise from 5 to 500 µg exosomes, for example from 5 to 200 µg.

The vesicles of the invention are obtained from genetically modified cells. As indicated above, the present invention in effect results from the finding that it is possible to insert recombinant molecules in some cells, by genetic route, and that these molecules

are then expressed in dense, functional manner in the exosomes.

To produce vesicles of the invention carrying recombinant molecules of determined composition, a first stage consists of inserting in a vesicle-producing cell such as defined above, genetic constructs which enable the expression of the chosen recombinant molecule(s).

The genetic constructs used for the production of cells may comprise, in general, a coding region placed under the control of a functional promoter in the cell used (expression cassette).

Generally, the promoter used is therefore a functional promoter in mammalian cells. It may be a viral, cellular or bacterial promoter for example. It may be a constitutive or regulated promoter, preferably allowing high-level protein expression in the cell. Among the promoters which may be used, mention may be made by way of example of the immediate early promoter of the cytomegalovirus (CMV), the promoter of SV40, the promoter of the thymidine kinase gene, in particular HSV-1 TK, the promoter of the LTR of a retrovirus, in particular LTR-RSV, or even a strong endogenous promoter of mastocyte cells. One particularly preferred embodiment comprises the use of the SRα promoter such as described in greater detail in the examples.

The coding region used is generally made up of a DNA, complementary, genomic or synthetic (modified

for example to comprise certain introns or to acquire the preferential use of codons). More generally, it is a cDNA. This nucleic acid may be obtained by any known molecular biology techniques, in particular by bank screening, amplification, synthesis, enzyme cuttings and ligatures.

Depending upon the type of coding region used, certain modifications may also be made to the construct. For example, it may be particularly advantageous in some cases to insert in the coding region a signalling sequence enabling the expression product to be addressed to a particular compartment of the cell, in particular towards a membrane compartment (internal, plasmic etc). This addressing signal may be positioned upstream (5'), downstream (3') or within the coding region. Preferably, the addressing signal is positioned at 3' of the coding region, more particularly in its cytoplasmic region, and in reading phase with the coding region. The use of an addressing signal may be particularly useful to promote accumulation of the expression product in or on the surface of a given intracellular compartment, especially in or on the surface of secretion vesicles. This embodiment is particularly adapted to the expression of a molecule such as a label peptide, an antigen, a MHC-I molecule, of even a receptor ligand. On the other hand, in particularly advantageous manner, the present application demonstrates that molecules of the human MHC-II can be expressed directly, without

adding a particular signal, in vesicles secreting mastocyte cells, even if xenogenic.

To implement the present invention, it is possible in particular to use as addressing signal a nucleic acid fragment having the sequence of part of the following genes : Lampl, CD63, LIMPII, Cdlc, FcγR. These genes comprise regions coding for addressing signals of the protein towards compartments of the endosome of the cells (Sandoval and Bakke, Trends in Cell Biol. 4 (1994) 292). An addressing signal which can be used in the present invention meets the formula G-Y-X-X-I for example, in which X represents any amino acid residue. One addressing signal particularly adapted to the present invention is made up of the peptide signal of the LAMPI protein having the sequence SHAGYQTI. Another type of signal allowing addressing towards membrane compartments comprises all or part of a protein transmembrane region.

The addressing of the expression product towards the cell compartments enabling this product to be present in the exosomes, may also be conducted by fusing the coding region to all or part of a region encoding a membrane or transmembrane protein, in particular a membrane or transmembrane protein expressed in the exosomes. In this context, one particular embodiment of the invention entails the insertion of a recombinant product in an exosome through the expression of this product in the producer cell, in the form of a fusion with a membrane or

transmembrane protein. One particular example of said protein is for example the recombinant protein of the MHC inserted in the producer cell, in particular a beta chain, preferably a class II beta chain of the MHC. Therefore, the results given in the examples show that such fusion enables the effective accumulation of any peptide of interest in an exosome, without affecting its properties nor those of the MHC molecule. This aspect of the present invention brings a new concept in the vectoring of recombinant products in an exosome and may be applied to any recombinant product inserted in any type of exosome. In this respect the invention therefore concerns any exosome comprising a recombinant molecule of fusion between a polypeptide of interest and an addressing signal. It may be an exosome produced from a mastocyte, dendritic or tumoral cell or also from a B-lymphocyte for example. The polypeptide of interest may be an antigen (or fragment of antigen) or any other biological product of interest. The addressing signal may be any peptide, polypeptide or protein having the property of directing the fusion product towards a membrane compartment, in particular an intracellular compartment, such as defined above. Advantageously it is a chain of a MHC molecule.

In one particular embodiment of the invention, these vesicles are produced by the insertion into the producer cell of a chimeric nucleic acid, coding for a fusion protein comprising the recombinant product bound

to the C-terminal end of a beta chain of a MCH molecule, preferably MCH class II.

In the constructs used, the coding region is bound in functional manner to the promoter such as to allow its expression in the cells.

Also, the constructs of the invention may advantageously comprise a region, positioned at 3' of the coding region, which specifies an end-of-transcription signal (polyA region for example).

The expression cassettes of the invention are advantageously part of a vector, of plasmid, viral, episomal, artificial chromosome type, etc. In this respect, said vector advantageously comprises a system enabling the selection of cells in which it is contained. In particular, the vectors advantageously comprise a gene coding for a product conferring resistance upon an agent, for example an antibiotic (ampicillin, hygromycin, geneticine, neomycin, zeocine, etc.). In one particular embodiment, each vector comprises a single expression cassette as described above. In this embodiment, the cells are therefore modified through the insertion of several vectors when several molecules are to be expressed in the vesicles (for example an α chain and a β chain of MHC-II). In this embodiment, each type of vector used advantageously comprises a different selection system, allowing the easy selection of multiple transfectants.

In another embodiment, a vector may comprise several expression cassettes such as defined above, for example one coding for an α chain and the other for a β chain of MHC-II.

The vectors used are preferably of plasmid type and comprise for example an origin of bacterial replication enabling their easy manipulation and *in vitro* production. Said vectors may in particular be constructed from plasmids of type pBR322, pUC, pBS, pSR, etc.

For the production of exosomes according to the invention, genetically modified cells are therefore used expressing the selected molecules. These genetically modified cells are prepared by inserting, in the chosen cells as defined above, the genetic constructs that are also described above.

The insertion of genetic constructs may be made in different ways, chiefly according to the type of cell used. Therefore, the transfer of nucleic acids may be made using any known technique such as electroporation, calcium phosphate precipitation, chemical agent (cationic peptide, polymers, lipids, etc), marking agent etc. In respect of viral vectors, the transfer is generally obtained by simple infection of the cells. The quantities of vector used may also be adapted by persons skilled in the art in relation to the type of transfer and cells used. In this respect one particularly effective method for the insertion of

nucleic acids in the mastocytes comprises electroporation of the vectors.

Also, when several constructs (vectors) need to be inserted in the cells, the latter may be transferred simultaneously or in sequential manner.

After transfer, the cells which have effectively incorporated the nucleic acids are selected and cloned on the basis of their resistance to a compound (e.g. antibiotic) through the resistance gene present in the transferred DNA. These cells may be used extemporaneously for the production of exosomes of the invention, or be stored for future use. In this respect, the cells may be stored at 4°C in a usual storage medium for a sufficient period to achieve several exosome production batches. The cells may also be stored in frozen form (in nitrogen for example) for later use. In this respect, it is therefore possible according to the prevent invention to form banks of exosome-producing cells having particular properties. In particular, it is possible, according to the invention, to form banks of cells expressing the main HLA types of MHC class II molecules. It will then be possible, depending upon the intended applications and upon HLA type, to choose from the bank the molecule producing cells of the corresponding MHC, without having to reconstruct these cells on a case by case basis.

In this respect, one particular object of the invention is in an exosome-producing cell such as

defined above, in particular a mastocyte cell, characterised in that it contains a recombinant nucleic acid coding for a molecule of the major Histocompatibility complex. The invention also concerns any exosome-producing cell such as defined above, in particular a mastocyte cell, characterised in that it contains a recombinant nucleic acid coding for a li invariant chain, particularly one modified to comprise an antigen peptide in the place of the CLIP region, or coding for a peptide enabling purification of the exosome.

More particularly, it is a mammalian cell, especially of animal origin, of a rodent in particular. It may also be a cell of human origin. In one particular embodiment, it is a cell line derived from a mastocyte, such as in particular a mastocyte line of a basophilic leukemia. By way of particular example, mention may be made of the RBL line, in particular RBL-2H3, the cells of the KU-812 line or HMC-1.

Preferably, the recombinant nucleic acid codes for an α chain and/or β chain of a class II molecule of the major Histocompatibility complex and/or for a class I molecule of the major Histocompatibility complex. In another embodiment, the cell comprises several nucleic acids respectively coding for an α chain and a β chain of a class II molecule of the major Histocompatibility complex.

With the present invention it is possible to produce, in simple reproducible manner, substantial

quantities of exosomes of known composition. For the production of exosomes, the genetically modified cells described above are cultured in an appropriate medium, and the exosomes are collected.

One particular object of the invention therefore lies in a method for producing an exosome containing a defined recombinant molecule, entailing the following stages :

a) culture of a mastocyte or mastocyte derived cell containing a recombinant nucleic acid coding for said defined recombinant molecule,

c) recovery of the exosomes produced by said cells, these exosomes containing said defined recombinant molecule.

Advantageously, the method of the invention also comprises an intermediate stage b) during which the cells are stimulated to induce and/or increase the secretion of exosomes.

Also, with the method of the invention it is possible to produce vesicles in which the defined recombinant molecule is exposed outside the exosome, or is included, wholly or in part, in the cytosolic fraction of the exosome.

As indicated above, in the method of the invention, the recombinant molecule may, for example, be a molecule of the major Histocompatibility complex, an antigenic molecule, a receptor ligand, a ligand receptor or a purification peptide, or any other

polypetide of interest. Also, as explained above, in some embodiments, the nucleic acid used in the method comprises in addition a region coding for an addressing signal towards the membrane compartments, in particular the internal secretion vesicles, of the mastocyte.

A further particular object of the invention lies in a method for producing a membrane vesicle, comprising :

- culture of an exosome-producing cell, containing a recombinant nucleic acid coding for a recombinant molecule of the MHC, in particular of class I or II, human in particular, and

- collection of the exosomes produced, optionally after stimulation of the exocytose.

In this respect, the invention also concerns a method for preparing an exosome containing a peptide-MHC complex of defined composition, characterised in that it comprises

- culture of an exosome-producing cell containing one or more recombinant nucleic acids coding for a defined recombinant molecule of the MHC,

- cell simulation to induce release of the exosomes,

- collection of the exosomes produced by said cells, these exosomes expressing on their surface said defined recombinant molecule of the MHC, and

- placing the exosomes in contact with the peptide(s)

For the implementation of the invention, the peptide(s) used may be synthesis peptides, peptide mixtures, cell extracts, for example a mixture of peptides extracted from tumour cells. The peptide(s) may be in isolated form, or purified, or, as indicated above, in a mixture. Also, after placing the exosomes in contact with the peptides, the exosomes can be isolated or purified using conventional methods.

In another variant, the invention concerns a method for preparing an exosome containing a peptide-MHC complex of defined composition, characterised in that it comprises .

- culture of an exosome-producing cell containing one or more recombinant nucleic acids coding for a defined recombinant molecule of the MHC and a nucleic acid comprising a region coding for a defined recombinant peptide,

- stimulation of the cells to induce release of the exosomes,

- collection of the exosomes produced by said cells, these exosomes expressing on their surface said defined recombinant molecule of the MHC associated with said recombinant peptide.

More particularly, in this process, the nucleic acid containing a region coding for the recombinant peptide, encodes a derivative of the invariant li

chain, in which the CLIP region has been deleted and substituted by said pepide. This embodiment ensures considerable specificity in the formation of the peptide-MHC complex.

In another variant, the nucleic acid comprises a region coding for the peptide and an addressing region towards the intracellular compartments. Also, the nucleic acid may contain several regions coding for one same or for different antigenic peptides.

Preferably, the producer cells used for the method are mastocyte or mastocyte derived cells. In this embodiment, the stimulation of cells to induce release of the exosomes is preferably by means of one or more calcic ionophors, or of IgEs.

In a particularly preferred embodiment, the producer cells used for the method are essentially free of molecules of the endogenous MHC.

A further object of the invention comprises a method for modifying the composition of an exosome, comprising

- insertion into an exosome-producing cell of a nucleic acid coding for a defined molecule, bound to an addressing signal in the membrane compartments, and
- producing exosomes from said cell.

With this method it is advantageously possible to produce exosomes expressing defined and varied recombinant molecules.

The exosomes of the invention may be used in numerous applications, for example as analysis, diagnostic, therapeutic or experimental tools. For example they may be used for analysis of the specific T antigen response ; for the study of receptor/ligand interactions of low affinity in which multimerisation of various partners is required in order to increase the avidity of these molecular complexes, thereby going beyond immunological fields of application ; for diagnosis and therapy and for the production of particular antibodies, in particular antibodies restricted to the MHC. These different applications and others are illustrated below.

a) Use for the production of antibodies.

One of the first applications of the exosomes of the invention lies in the production of antibodies. Given the defined composition of the exosomes of the invention, it is possible to produce antibodies having determined specificity. Also, as shown in the examples, the exosomes of the invention have very strong immunogenic properties, in particular on account of the high density of the MHC-peptide complexes on their surface, their functionality and their efficient presentation to the immunity system.

The antibodies produced in this way may be polyclonal or monoclonal. They may be prepared using conventional immunology techniques, comprising animal immunisation, collection of sera (polyclonal antibodies) and/or the fusion of spleen lymphocytes

with myeloma cells which do not produce immunoglobulins (to generate monoclonal-producing hybridomes).

A further object of the invention therefore concerns antibodies or fragments of antibodies produced by immunisation with exosomes such as described above. The fragments of antibodies may for example be Fab, (Fab')2, ScFv fragemnts etc, and more generally any fragment maintaining the specificity of the antibody. In particular, the invention concerns a method for preparing antibodies, comprising immunisation of an animal with an exosome such as described above, carrying a defined peptide-MHC complex, and recovery of the antibodies and/or cells producing antibodies or involved in the immunity response. Advantageously, with the method of the invention it is possible to produce monoclonal antibodies, in particular restricted to the MHC, that is to say specific to the MHC-peptide association. Preferably, in the method of the invention, exosomes essentially free of endogenous MHC molecules are used, which express recombinant MHC-peptide complexes, and which are produced from a cell that is autologous vis-à-vis the animal in which immunisation is made. Therefore, as shown in the example, with this method it is possible, with no need for an additive, to obtain powerful antibodies directed against the peptide, in particular antibodies restricted to the MHC, that is to say specific to the peptide in its conformation associated with the defined molecule of the MHC. Such antibodies are particularly

advantageous on experimental, diagnostic and therapeutic levels. Also, the antibodies of the invention may be labelled by any known technique (enzymatic, fluorescent, radioactive, etc), using methods known to persons skilled in the art.

b) Diagnostic applications

The exosomes and antibodies of the invention have advantageous properties for diagnostic use.

For example, the antibodies or fragments of antibodies obtained according to the invention, may be used for any diagnostic applications, for detection in a biological sample of the presence of corresponding specific antigens through the use of different conventional techniques, such as flow cytometry, immunohistochemistry or immunofluorescence for example. In the particular case of antibodies restricted to the MHC, they advantageously allow the detection of corresponding MHC-peptide complexes, and hence the diagnosis of corresponding pathologies. These antibodies may in particular be applied to the diagnosis of pathologies involving a defect in response or an inappropriate response of the immunity system in order to determine the expression of an antigen, previously defined, in a form recognisable by the T-lymphocytes. For example, and in non-exhaustive manner, the following diagnoses can be considered :

- tumour pathologies in which the detection on tumour samples of different peptides derived from proteins such as p53, Her2, MAGE, BAGE, MART, GP100

associated with class I molecules of the MHC, can enable the phenotyping of the tumour and facilitate choice of treatment ;

- viral diseases at a pre-infectious or latent stage, in which the virion cannot be detected (hepatitis, HIV, CMV or other virus infection)

- autoimmune disease such as multiple sclerosis, autoimmune diabetes, autoimmune thyroid dysfunction, rheumatoid arthritis, lupus erythematosus, in which the detection of MHC molecules showing peptides derived from autoantigens may be a precursor indication of further development of the disease.

The exosomes of the invention may also be used to detect specific partners of a protein molecule in a biological sample. Therefore, exosomes of the invention carrying MHC-peptide complexes can be used to detect T-lymphocytes specific to these complexes in biological samples, for example in different pathological situations, in particular in the above-mentioned pathologies. In this respect, the exosomes may be labelled by any labelling system known to persons skilled in the art (enzymatic, fluorescent, radioactive, etc) to allow their detection in biological samples.

In one particular embodiment, the invention therefore relates to the use of labelled exosomes, especially fluorescent labelled, such as described above for the detection of T-lymphocytes specific to antigen peptide-MHC complexes in a biological sample.

The biological sample may be any sample of blood, serum, tissue, tumour, biopsy, skin, urine, etc. Also, the biological sample may be pre-treated for example to dissociate the cells, amplify the cells in culture, prepare membrane fractions, etc. Advantageously, the biological sample is derived from a human organism. For this purpose, the invention also concerns a method for the detection of the presence of T-lymphocytes specific for antigen-MHC complexes in a biological sample, comprising the placing in contact of said sample with a labelled exosome such as defined above, containing said antigen-MHC complex, and detection of the labelling of the T-lymphocytes in said sample.

Moreover, the detection of these T-lymphocytes not only enables the detection and therefore the diagnosis of a physiopathological condition, but it also enables follow up of the effectiveness of immunisation protocols for example and the status of the immunity response at different stages of the disease and hence to assess the efficacy of treatment given.

In one particular application, the exosomes of the invention carrying a TcR receptor are used for the detection of specific peptide-MHC complexes of this receptor in a biological sample.

Also, the fluorescent exosomes of the invention carrying any type of protein of defined composition also form fluorescent probes enabling the detection of potential receptors. The new field opened up by

exosomes can therefore be generally extended to the *in vivo* detection of any protein/protein interaction of low affinity. A further purpose of the invention is therefore the use of exosomes, preferably labelled, especially by fluorescence, such as described above,

- for the detection of specific receptors of a protein molecule in a biological sample. In this embodiment the exosomes used therefore comprise on their surface said biological molecule of defined structure,

- for the detection of the presence of a ligand in a biological sample. In this embodiment, the exosomes used therefore contain, on their surface, a specific receptor of said ligand.

c) Therapeutic applications

The restricted antibodies or fragments thereof are potentially able to inhibit the interaction between the receptor of a T-lymphocyte and the MHC-peptide complex for which it is specific. In parallel, the exosomes carrying on their surface a single type of MHC-peptide complex may, by interacting with the T-lymphocytes specific to these complexes, enter into competition with their natural ligands, the T-lymphocytes, and lead to their inactivation.

Restricted antibodies and exosomes may therefore be used in any situation in which it is required to reduce or suppress an immunity response

mediated by T-lymphocytes which proves to be noxious for the organism, such as is the case for example in:

- organ transplants or bone marrow grafts in which it is sought to neutralise the host response to the graft, generally by means of strong doses of immunosuppressors ;

- autoimmune diseases or viral pathologies during which the T CD8 or CD4 immunity response chronically leads to tissue destruction ;

- allergies and asthma.

In these types of pathologies, the exosomes of the invention expressing on their surface a defined peptide-MHC complex, which is known to be involved in the development of the pathological condition, can therefore be used to block the development of the immune response and therefore the development of the pathological response.

The exosomes of the invention carrying MHC-peptide complexes may also be used to amplify (expand) *ex vivo* the population of cytotoxic T-lymphocytes. Used directly from blood samples, they can therefore form the basis of cell therapies against different cell targets. Hence the exosomes can be used to sort T cells of varied combinations specific to complexes expressed by cells which represent a therapeutic target, such as tumour cells or virus-infected cells. A further object of the invention is therefore the use of the exosomes described above for the clonal amplification and/or

stimulation of cytotoxic and/or auxiliary T-lymphocytes. The invention also concerns a method for the *ex vivo* clonal amplification (or expansion) of T-lymphocytes, in particular cytotoxic lymphocytes, which entails placing a biological sample containing T-lymphocytes in contact with exosomes such as described above, containing a defined peptide-MHC complex, collection of the specific T-lymphocytes and their amplification. This method is particularly advantageous for the clonal amplification of cytotoxic T-lymphocytes specific to complexes between MHC molecules and peptides of tumoral or viral antigens.

A further application of particular interest of the vesicles of the invention is the transfer of molecules towards cells. Through their composition, the vesicles of the invention are able to play a vector role in the transfer of molecules towards cells, *in vitro*, *ex vivo* and *in vivo*. In this respect, the invention concerns the use of exosomes such as described above, containing a substance of interest, for the preparation of a composition intended for the transfer of said substance into a cell. Advantageously, it is an exosome containing a ligand receptor or receptor ligand on its surface, making it possible to orient the transfer towards one or more chosen cell populations. The invention also concerns a method for the transfer of a substance into a cell, *in vitro*, *ex vivo* or *in vivo*, which entails placing said cell in contact with a vesicle of the invention containing said

substance. More preferably, the vesicle used also expresses a ligand receptor and the method of the invention enables the oriented transfer of the substance towards cells expressing the corresponding ligand. For *in vivo* implementation, the vesicles of the invention are administered (preferably to a mammal, man in particular) by any conventional route (intravenous, intraarterial, intramuscular, subcutaneous injection, etc.). For *in vitro* or *ex vivo* use, the cells are contacted by incubation in an appropriate device (dish, flask, pouch, ampoule, etc.) preferably under sterile conditions. The parameters of the contact-making stage (amount of vesicle, contact time, temperature, medium, etc.) may easily be adjusted by persons skilled in the art in relation to the set purpose and the teaching of the present application.

d) Applications in research areas

These evidently concern all the applications mentioned above for the analysis of molecular mechanisms in antigen presentation through the use of antibodies able to detect and analyse the different stages of formation of MHC-peptide complexes in different normal or pathological situations.

They also concern the analysis and molecular characterisation of T-lymphocyte populations able to recognise a determined MHC-peptide complex through the use of fluorescent exosomes and their ability to detect

In these different applications (diagnostic, therapeutic, experimental, production of T-lymphocytes, etc.), the exosomes of the invention may be implemented either as such, or in immobilised form on a support medium. Therefore, the results given in the examples show that it is possible to fix exosomes on support media without deteriorating their functional properties, in particular their antigenic specificity for example. In this respect, one particular purpose of the present invention lies in a composition containing exosomes immobilised on a support medium. The support medium is preferably a solid or semi-solid support such as a bead, filter or similar. It is preferably a support in plastic material, of polymer type, for example latex beads or magnetic beads. Evidently any other synthetic or biological material can be used provided that it does not cause any substantial deterioration in the qualities of the exosomes or cells. Advantageously beads having a diameter of 1 to 10 µm are used, for example 2 to 5 µm. The immobilisation of the exosomes on support media is advantageously obtained by covalent bonding, for example by activation with an aldehyde, or any other chemical binding reagent. Generally the immobilisation of the exosomes is made by incubation of the exosomes with the support in solution, under conditions allowing fixation, then the supports are collected by centrifuging. The functionalised supports obtained in this way may be used to characterise the exosomes or to

detect or amplify T-lymphocytes *in vitro* as will be described in detail in the experimental section.

Other aspects and advantages of the present invention will be seen on reading the following examples which are to be considered as illustrative and non-restrictive. Also all the publications cited in this application are incorporated herein by reference.

Keys to figures

Figure 1. Production of functional MHC-DR1-HA peptide complexes in the RBL2H3 line.

A. Analysis by flow cytometry of the surface expression of DR1 molecules of the human MHC-II before (left) and after (right) transfection of the cDNAs coding for the α and β chains of DR1 in the RBL 2H3 line. The DR1 molecules are detected by the L243 antibody (black line) itself developed by a goat serum antimouse-IgG coupled to the FITC.

B. Surface expression of DR1 in the line expressing an invariant chain (liHA) in which the CLIP peptide has been replaced by the 308-319 peptide derived from flu virus hemagglutinin. The DR1 molecules are detected on the RBL DR1 liHA line and a B-lymphocyte transformed by EBV (Hom2) of same haplotype by the L243 antibody (black line).

C. Stimulation of T-lymphocytes specific to the DR1-HA complex by the RBL line expressing this complex or B-EBVs of the same haplotye. Lines RBL DrlliHA and B-EBV Hom2 were diluted in culture dishes with a T-

lymphocyte specific for the DR1 HA complex. Line B-EBV Hom2 was also incubated in the presence of a saturating concentration (10mM) of the HA peptide. The production of IL2 in the culture supernatants is used to evaluate the stimulation of the THA lymphocytes (T-lymphocytes specific to the HA peptide). IL2 is measured by means of a tritiated thymidine incorporation test in the CTLL2 line whose proliferation is IL2-dependant.

D. Analysis of HA peptide saturation of the RBL DR1 liHA line. Cells Hom2 and RBL DR1 liHA were incubated (100 cells per dish) in the presence of increasing concentrations of the HA peptide and THA lymphocytes. Stimulation of the lymphocytes was assessed as previously.

Figure 2 : Accumulation of DR1 molecules in a secretion compartment of RBL2H3.

A. Analysis of the intracellular accumulation site of DR1 molecules in RBL 2H3. Cells RBL DRLHA were fixed with 0.5% glutaraldehyde then permeabilised with 0.05% Saponine. The DR1 molecules and the invariant chain were respectively detected with antibodies L243 and PIN1 then a donkey serum antimouse-IgG coupled to the FITC. Serotonine was detected using specific rabbit serum developed with a donkey serum antirabbit-IgG coupled to Texas red. The images were obtained by confocal microscopy (Leica). Section thickness was 0.5 micron.

B. Purification of exosomes of RBL DrlliHA. After washing in DMEM, the cells were incubated for 30

minutes in the presence of 1 mM ionomycine at 37°C. The exosomes were purified by differential ultracentrifugation from the cell supernatant. The exosome residue, replaced in suspension in PBS, was separated (5 mg) by SDS-PAGE then transferred onto a Nylon membrane. The β chain of DR1 was detected with the monoclonal antibody IB5 for exosome preparation and for control in the lysates of RBLDRLHA and Hom2 cells (equivalent to 10^5 cells per dish) migrated under the same conditions.

Figure 3 : Use of exosomes for the production of antiDR1 HA antibodies

A. Increasing dilutions of sera from mice immunised with the exosomes were incubated with RBL cells expressing (right) or not expressing (left) the DR1 HA molecules. The labelling obtained was analysed by flow cytometry.

B. Sera from rats (diluted to 1/100) immunised with the exosomes were incubated with RBL cells expressing or not expressing (left) the DR1 HA molecules. On the right, the cells expressing DR1 were or were not previously incubated for two hours at 37°C with 10 mM of the HA peptide, then with the same dilution, with serum from immune rats.

C. The spleen of the immune rat was fused with the X63A8 line under conventional production conditions for monoclonal antibodies. The supernatant of the different hybridomes was tested by immunofluorescence

on RBL2H3 cells either expressing or not expressing the DR1 or DR1 HA molecules. Clones a40, b82 and a15 are representative examples of the antibodies obtained.

Figure 4 : Use of exosomes for the detection of T-lymphocytes specific to the DR1 HA complex.

A. Cells RBL DR1 liHA were incubated in the presence of 5 mM "Green Tracker" (fluorescent lipid accumulating in the lyosome compartments of cells) for 30 minutes at 37°C, then washed and re-incubated for one hour at 37°C in the absence of a fluorescent tracer. The cells were fixed (3% paraformaldehyde), then analysed under confocal microscopy.

B. In parallel, exosomes DR1 HA were purified from the cells described in A. The fluorescence present in the samples was quantified with a fluorimeter and directly visualised under confocal microscopy.

C.D. The fluorescent exosomes DR1 HA were incubated at 50mg/ml with THA lymphocytes specific to the DR1 Ha complex or with TH30 lymphocytes specific to another complex (D) for tw hours at 37°C in the presence of azide to block internalisation. The fluorescence of the cells was evaluated by flow cytometry.

Figure 5 : Production of exosomes carrying class II MHC molecules.

A. The expression of class II molecules IAb is detected by the monoclonal antibody Y3P and analysed by flow cytometry. The transfectants obtained in cell

RBL2H3 express levels of class II molecules recognized by Y3P similar to a B414 control B-lymphoma.

B. Western blot analysis of the expression of molecule IAb in RBL. 10 mg of a cell lysate and a preparation of exosomes derived from cell RBL IAbIi were analysed by Western blot with rabbit serum specific to the cytoplasmic region of chain a of molecule IA.

C. Analysis by flow cytometry of the composition of exosomes. Latex beds were coated either with fetal calf serum (FCS) or with exosomes derived from cells RBL 2H3 (exos RBL) or with transfectant of this cell using murine class II molecules IAbIi (Exos IAbIi) or human DR1IiHA (exos DR1IiHA). Molecule CD63 of the rat is detected with the antibody AD1, molecules IAb with antibody Y3P, while molecules DR1 are detected by antibody L243. These different antibodies are developed by secondary antibodies coupled to phycoerythrine.

Figure 6 : Morphological characterisation of exosomes produced by RBL-2H3.

A. Cells RBL-2H3 transfected with HLA-DR1 were fixed with paraformaldehyde. Ultrafine frozen sections were prepared and immunolabelled with polyclonal antibodies directed against molecules HLA-DR. These antibodies are visualised with protein A coupled to 10 nm particles of colloidal gold. The class II molecules are chiefly detected in the compartments filled with membranes of vesicle appearance. Bar : 250 nm.

B.C. Morphological characterisation of the exosomes secreted by the RBL-2H3 cells. The exosomes are fixed with 2% paraformaldehyde in 0.2M phosphate buffer pH 7.4 (PB buffer) and deposited on electronic microscope plates covered with a film of carbonated formvar. The exosomes are either contrasted and coated in a 4% solution of uranyl acetate and methylcellulose or (b) immunolabelled with antibodies directed against class II molecules before coating (c). As in figure (a), the antibodies are visualised with protein A coupled to 10 nm particles of colloidal gold. Bars : 250 nm.

Figure 7 : Manipulation of the internal composition of exosomes, insertion of a recombinant protein.

A. The expression of class II DR1 molecules is detected by the monoclonal antibody L243 and analysed by flow cytometry. The transfection of molecules in cell RBL2H3 induces the expression of levels of class II molecules recognized by Y3P similar to a B414 control B-lymphoma.

B. Western blot analysis of the expression of molecule DR1 GFP in RBL. 10 µg of a cell lysate and 20 µg of a preparation of exosomes derived from cell RBL DR1 GFP were Western blotted with a couple of GFP-specific monoclonal antibodies.

C. Analysis by flow cytometry of the composition of exosomes. Latex beads were coated either

with fetal calf serum (FCS) or with exosomes derived from cells RBL DR1GFP. The DR1 molecules are detected by the L243 antibody and.by the L243 antibody and secondary antibodies coupled to phycoerythrine while the presence of GFP is detected directly in channel FL1.

Figure 8

A. Binding of fluorescent exosomes by specific T-cells. Exosomes produced from RBL DR1 liHA cells labelled with green cell tracker were incubated in the presence of two types of T-cells : THAs which have a specific TCR for the HLA-DR1/HA complexes, and wild T-Jurkat not containing this receptor. The fluorescent exosomes were incubated for 3 hours at 37°C with two lines of T-lymphocytes and the resulting labelling was then FACS analyzed.

B. Acquisition of an exosome tracer with specifically labelled T-cells. The same labelling as in A was made but the binding of exosomes (not labelled with green cell tracker) to the T-cells was detected with a mouse monoclonal antibody AD1 antirat CD63, subsequently developed with donkey antibodies antimouse IgG labelled with phycoerythrine (Jackson Immuno Research, West Grove, PA). The same labelling was conducted in parallel on T-cells which had not been exposed to the exosomes.

C. Stimulation of Lymphocytes by exosomes. The exosomes purified from DR1GFP cells were crosslinked on latex beads prepared in the same manner as for flow

cytometry but were washed in a complete medium. Each bead residue was collected in 100 µL, 50 µL being placed in the first well of a 96-well multidish and 50 µl diluted in two-fold increments. The T-cells (T-Jurkat and THA1.7) were adjusted to 106 cells/mL and 50 µL was placed in each well in the presence or absence of 5 µM HA307-319 peptide. The culture dish was placed in an incubator (37°C, 5% CO2, H2O) for 20 hours, then the supernatant was collected and the IL2 concentration was evaluated with a CTL.L2 test.

Figure 9 : Characterization of HMC-I cells and exosomes

A. Analysis of HMC-I cells by flow cytometry, bold continuous line: cells alone ; light continuous line: antimouse antibody-FITC alone ; dense dotted line: specific antibody + antimouse-FITC.

B. Analysis of HMC-I exosomes cemented to latex beads by flow cytometry, bold continuous line: latex beads-HMC-I exosomes alone ; light continuous line: antimouse antibodies-FITC alone ; dense dotted line: reference latex beads-SVF + specific antibody + antimouse-FITC ; open-dotted line : exosome-latex beads + specific antibody + antimouse-FITC.

C. Western blot analysis of a lysate of MHC-1 cells compared with their exosomes ; 10 or 3 µg proteins per well ; HC10: 1/10 supernatant ; 1B5: 1/10 supernatant ; CD63: 5 µg/l , Lamp1: 2 µg/mL ; H68.4: 1/10 supernatant. The exosomes appear enriched in CD63,

Lamp1, TfR. The absence of class II MHC both in the lysate and in the exosomes confirms cytofluorometric analysis. The proportion of class I MHC appears identical for the cell lysate and for the exosomes.

Material and Methods

Cells

The cells used for the production of exosomes in the experimental part are murine or human mastocyte cells. More particularly, a tumoral line of basophils of mucous mastocyte phenotype, designated RBL-2H3, was used (Barsumian et al., Eur. J. Immunol. (11 (1981) 317), and a line of immature human mastocytes (MHC-1). Other mastocyte cells, particularly in lines, may be used such as lines derived from RBL cells (Rat Basophilic Leukemia) filed with ATCC under number CRL1378 (Kulczycki et al., J. Exp. Med. 139 (1974) 600).

T-lymphocyte lines able to recognise a particular antigen in a human MHC-II context (DR1) were also used. In particular, the Jurkat line transfected with cDNA coding for the receptor of T ("T-HA") cells specific to peptide 306-318 of the flu virus hemagglutinin in association with HLA-DR1 (Sidhu et al., J. Exp. Med. 176 (1992) 875). The line of human B cells transformed by the Epstein Barr virus (line Hom-2) was used as control for the restricted response to HLA-DR1.

The cells were cultured in DMEM medium (Gibco BRL), RPMI, or "CLICK" : RPMI medium supplemented with 10% fetal calf serum (Sigma), 1 mg/ml penicillin-streptomycin, 1 mg/ml glutamine, 5 mM sodium pyruvate and 50 mM β-mercaptoethanol. Any other medium adapted to the culture of eukaryote cells, mammalian in particular, may evidently be used.

The cells were cultured mainly in a culture flask of 25 or 15 cm3. Since the RBL-2H3 cells are adherent cells, they are lifted from their support using Trypsin-EDTA (Seromed). In order to produce the latter in large quantities, it is also possible to culture them in a "spinner" to a density of 10^6 cells/ml.

Plasmids

To genetically modify mastocyte cells, the following genetic constructs were made.

The cDNAs coding for the human HLA-DR1 α chain (Larhammer et al., Ceil 30 (1982) 153), the human HLA-DR1 β chain (Bell et al., PNAS 82 (1985) 3405) and the invariant human chain p33 li (Ciaesson et al., PNAS 80 (1983) 7395) were isolated. The cDNA coding for the invariant chain p33 li was then modified by PCR to replace the region coding for the CLIP peptide (residues 87-102) by a restriction site. With this cDNA it is possible to insert, in lieu and stead of the CLIP peptide, any cDNA fragment of interest coding for an antigenic peptide (Stumptner et al., EMBO J. 16 (1997)

5807). In one precise example, a DNA fragment coding for the HA308-319 peptide of flu virus hemagglutinin was inserted into this cDNA coding for a chimeric Ii polypeptide (HA308-319).

The nucleic acids described above were then cloned, separately, in the pSRα plasmid, under the control of the SRα promoter (Takebe et al., Mol. Cell Bio. 8 (1988) 466). Each of the plasmids was then modified such as to incorporate a different resistance gene, allowing selection for each of the plasmids and hence for each of the chains ; chain α with the resistance gene to neomycin ; chain β with the resistance gene to hygromycin, and the invariant chain with the resistance gene to zeocine.

Transfections

To insert the different nucleic acids in the mastocyte cells, the corresponding plasmid vectors were linearised with the ScaI restriction enzyme. 50 µg of each plasmid were linearised, then ethanol precipitated, and the residues were re-suspended in the presence of RBL-2H3 cells at a concentration of 1.10^7 cells/ml. Stable transformants were obtained by electroporation of 5.10^6 cells using a "gene pulser" (Bio-Rad, Richmond, CA) under the following conditions : 260V, 960 µF. 72 hours after electroporation, the transfectants were selected by culture in a selection medium comprising 250 µg/ml G418 (Généticine, Gibco) 1 mg/ml hygromycin and 500 µg/ml zeocine. After 8 days'

culture in the selection medium, 60 to 90% of the cells present are transfected. The transfectants are then seeded in a Petri dish with selection medium at a concentration enabling the onset of individualised adherent colonies. The clones obtained in this way were collected and placed in culture separately. These clones may be stored in frozen form for future use.

Antibodies

Y3P (MHC-I (IA) is a mouse monoclonal antibody which recognises the IAb aβ complex (Janeway et al., 1984). Anti-IA a is a rabbit serum directed against the cytoplasmic part of the a chain of IA. Anti-GFP is a mixture of two monoclonal antibodies (clones 7.1 and 13.1) directed against "green fluorescent protein" marketed by Boehringer Mannheim. In flow cytometry experiments, the second antibodies used are F(ab')2 fragments, coupled to Phycoerythrine, produced by donkeys and directed against mouse IgG (H+L) (Jackson Immunoresearch Laboratories).

Beads

The latex beads : Surfactant-free white aldehyde/sulfate latex, D : 3.9 µm, Interfacial Dynamics Corp., Portland, Or. USA:

Electronic microscopy

The RBL-2H3 cells transfected with HLA-DR1 were fixed with 2% paraformaldehyde in 0.2M phosphate buffer pH 7.4 (PB buffer). After fixation the cells were washed with PB buffer-50mM glycine, then coated in 10%

gelatine. After solidification blocks were prepared, infused in 2.3M sucrose and frozen in liquid nitrogen. Ultrafine frozen sections were prepared and immunolabelled with polyclonal antibodies directed against the HLA-DR molecules. These antibodies are visualised with A protein coupled to 10 nm particles of colloidal gold.

The exosomes are fixed with 2% paraformaldehyde in 0.2M phosphate buffer pH 7.4 (PB buffer) and deposited on the plates of the electronic microscope covered with a carbonated formvar film.

The exosomes are either contrasted and coated in a 4% solution of uranyl acetate and methylcellulose, or immunolabelled with antibodies directed against class II molecules before coating. The antibodies are visualised with A protein coupled to 10 nm particles of colloidal gold.

Results

I - Production of DR1 HA exosomes

1.1 Construction and characterisation of genetically modified producer cells

In order to produce, in controlled manner, exosomes carrying MHC-peptide complexes of defined composition, chains a and b of class II MHC molecules, DR1, were expressed in the RBL2H3 lo mastocyte line, derived from rat basophilic leukemia. For this purpose, two vectors respectively carrying a nucleic acid coding for each chain, under the control of the SRa promoter,

were transfected simultaneously in the cells (see material and methods). The results obtained by flow cytometry show that the transfected cells effectively express the DR1 molecules (Figure 1A).

These RBL-2H3 DR1 cells were then made susceptible to a given peptide, of precise composition, in order to generate MHC-peptide complexes of defined composition. For this purpose different techniques can be considered. In a simple embodiment, the peptide can be incubated directly with the exosomes. In another variant, a nucleic acid coding for the peptide can be inserted in the cells such as to also express this peptide. In this particular example of implementation, to produce a presenting cell containing a single antigenic specificity, the chosen antigenic peptide was inserted in the cells in the form of a genetic fusion with the invariant human li chain. More particularly, the CLIP peptide of the invariant chain was replaced by the sequence of the chosen peptide, derived from flu virus hemagglutinin (HA 308-319) known to bind to the DR1 molecule. This construct (liHA) was transfected in the cells under the conditions described in Material and Methods. The hybrid chain expressed in the RBL-2H3 DR+ cells enabled the construction of cells which express DR1 molecules recognised by the L243 antibody at a level similar to a control B-EBV (Hom2) line of DR1 haplotype (Figure 1B). These results consequently show that the mastocyte cells of the invention

effectively express a human, functional peptide-MHC complex of pre-determined, controlled composition.

The functional character of the peptide-MHC complexes expressed by the cells of the invention was confirmed by a stimulation test of T-lymphocytes specific to the DR1-HA combination carried by the cells. For this purpose, the cells of the invention were incubated in the presence of THA lymphocytes, and stimulation was determined by measuring the interleukin-2 released into the supernatant, by a growth test of a IL-2 dependent cell line. The control used was a line of B-lymphocytes transformed with EBV (Hom2), of DR1 haplotype, pulsated with a saturating concentration (10mM) of the HA peptide.

The results obtained are given in Figure 1C and 1D. They show that the mastocyte cells of the invention express a DR1-HA complex able to stimulate a T-lymphocyte specific to this combination. They also show that the stimulation obtained in the presence of the cells of the invention is more effective than that produced by the control cells (B-EBV of DR1 haplotype) pulsated by a saturating concentration (10mM)of the HA peptide. Finally, the results obtained show that the DR1 molecules appear only to present the HA peptide since the addition of a saturating concentration of the peptide does not significantly increase the capacity of the RBL DR1 liHA cells to stimulate a THA lymphocyte (Figure 1D).

All these results therefore demonstrate the functional character of the peptide-MHC complexes produced. They also illustrate the specific character of the cells obtained and therefore the specific character of the method of the invention which enables cells (and exosomes) to be obtained which carry molecules of defined, controlled composition.

1.2 Production of functionalised exosomes

Immunofluorescence tests showed that the recombinant MHC-peptide complexes (DR1 HA) accumulate in the secretion granules of the RBL-2H3 cell line. Figure 2A gives evidence of the co-localisation of the DR1 molecules with serotonine in vesicle intracellular structures.

The possibility that functional exosomes may be released by these cells was therefore examined. For this purpose, the cells were cultured in the presence of an calcic ionophor, and the production of membrane vesicles was followed up. More particularly, the cells were centrifuged at 300 g for 5 minutes at room temperature. On each cell residue, a solution of calcic ionophor (1 mM ionomycine) was added (approximately 300 µl) and incubation was continued for 30 minutes at 37°C. Exocytosis was halted by rapid cooling on ice and the addition of 300 µl cold 1mM PBS-EGTA solution. The cells were then centrifuged at 300 g at 4°C for 5 minutes. The supernatants were recovered and re-centrifuged, firstly for 5 minutes at 1200 g, then 5 minutes at 10000 g, and finally 1 hour at 70000 g.

After this differential centrifugation, the residues (including the exosomes) are collected and solubilized in a buffer solution (30 µl Laemmlî-DDT buffer (IX or 2X). A fraction of the residues is also solubilized in a lysis buffer to determine the protein concentration. The exosome solutions may be separated by gel migration (12% polyacrylamide mini-gel) at 20 mA then transferred onto Immobilon. Analysis of the exosomes is then conducted by Western blotting with specific antibodies of the different chains of class II MHC molecules.

The results obtained show that the exosomes can be released from the RBL-2H3 line in substantial manner, after stimulation by an appropriate agent. These exosomes may be isolated and purified for example by differential ultracentrifugation for the preparation of exosome compositions. Finally, the results given in Figure 2B show that these exosomes are functional. Western blotting analysis demonstrates that the exosomes obtained express the different recombinant chains of class II molecules of the MHC. In addition, these results also show the high density of the peptide-MHC complexes on the surface of the exosomes of the invention.

The following examples illustrate in particular the use of DR1 HA exosomes for the production of antibodies specific to this combination, and the capability of the DR1 HA exosomes to bind T-lymphocytes specific to this same combination.

2. Generation of antibodies specific to the DR1 HA complex

This example illustrates the use of exosomes of the invention for the production of antibodies, in particular so-called "restricted" antibodies, that is to say specific to the antigenic peptide in association with the MHC molecule. This example illustrates in particular the very strong immunogenic property of the exosomes of the invention since they enable the production of antibodies without any additive.

The exosomes purified from the supernatant of the RBL DR1 HA cells (example 1) were re-suspended in PBS. These exosomes were then used to immunise Balb/c mice or LOU rats without any additive in accordance with the following protocols :

- The mice were injected by subcutaneous route with 10 µg exosomes, in two injections separated by a time interval of 3 weeks, then 30 µg by intraperitoneal route and finally 30 µg by intravenous route 3 days before serum collection.

- The rats were injected with exosomes by intraperitoneal route (10µg) in two injections separated by time interval of 3 weeks, then by intravenous route (50µg) three days before serum collection.

As shown in Figure 3A, the sera collected from the immunised mice showed very strong reactivity against the RBL line whether expressing or not

expressing the DR1 HA complex, but which was detectable down to a serum dilution of thirty thousandths only for cell DR1 HA.

As shown in Figure 3B, the sera of immunised rats, in surprising manner, showed reactivity against the RBL line expressing the DR1 HA complex whereas the same sera reacted with lower intensity against the initial RBL-2H3 line. Also, the addition of the HA peptide to DR1 expressing cells(RBl-2H3 DR1) brought a significant increase in the reactivity of the antisera produced (Figure 3B).

These results therefore show that the exosomes of the RBL line are able to induce an antibody response which, in unexpected manner, is mainly directed in the rat against DR1 HA complexes.

The spleens of immune rats were fused with cells of the X63A8 line. The hybridomes obtained were sorted by clonal dilution using conventional immunology techniques, then selected by immunofluorescence for the specificity of the monoclonal antibodies produced. Different monoclonal antibodies were obtained in this way, some directed against proteins of the RBL line, others against monomorphic determinants of human class II molecules of DR1 haplotype, and finally others against the complex made up of the DR1 molecules associated with the peptide derived from the HA protein of the flu virus (Figure 3C). These latter monoclonal antibodies form restricted antibodies and therefore

have particularly advantageous properties for diagnostic or therapeutic applications .

3 -Detection of T-lymphocytes specific to the DR1 HA complex.

This example illustrates the use of exosomes of the invention for the detection of specific T-lymphocytes in a biological sample. This example also shows how the exosomes may be used to select and amplify a population of particular T-lymphocytes intended in particular to be re-injected into an individual (cell therapy). This approach may evidently be extended to the use of the restricted antibodies described in example 2, and to the detection of any ligand-specific receptor.

For the implementation of this application, labelled exosomes were produced. For this purpose, before purification of the exosomes of the DR1 HA line, the latter was incubated with a fluorescent tracer which strongly accumulates in the exosomes contained in the secretion granules. The tracer used "Green Tracker" is a fluorescent lipid which accumulates in cell lysosomes. Analysis of the cells under confocal microscopy, after fixation, shows the presence of fluorescent labelling in the secretion granules of the cells (Figure 4A). The fluorescent exosomes were then produced and purified from these cells under the conditions described in example 1. Thus made fluorescent, these exosomes (Figure 4B) were used to detect, in a biological sample, the presence of T-

lymphocytes specific to the DR1 HA combination (THA lymphocytes). it is evident that any other labelling may be used within the scope of this invention, applied either to the producer cells or to the exosomes produced

For this purpose, the exosomes were incubated in the presence of a sample of THA lymphocytes and TH30 lymphocytes specific to another complex. The results obtained by flow cytometry show that the exosomes expressing the DRLHAs bind, in specific manner, to the THA lymphocytes (Figure 4C) whereas these same exosomes are incapable of recognising lymphocytes having another specificity (Figure 4D). These results show the unique, unexpected capacity of the exosomes produced by the mastocyte line of the invention to detect T-lymphocytes specific to this same complex. This application may be implemented using any type of biological sample.

Also, this technology can be applied in the same manner to the production and use of exosomes expressing MHCI-peptide complexes. With the present invention it is therefore possible to detect, on the surface of presenting cells, even tumour cells, the presentation by MHC class I and class II molecules of peptides derived from antigens expressed by the tumours. The present invention therefore also enables the detection even the purification of T-lymphocytes able to recognise these same complexes. They can therefore be used to amplify a population of T-lymphocytes specific to a particular peptide-MHC

complex, for example a population of CTL lymphocytes for the purpose of their therapeutic use. Different immunotherapy approaches for cancers or viral infections for example have been developed on the basis of collecting lymphocyte samples from an individual and on the *in vivo* expansion of particular clones of T-lymphocytes specific to an antigen involved in the pathology (tumoral or viral antigen for example). These amplified clones are then re-administered to the individual as a therapeutic agent. The prevent invention brings much greater ease in the selection and amplification of specific clones of T-lymphocytes, and therefore in the potential and implementation of these therapeutic approaches.

4 - Production of exosomes carrying class II molecules of the murine MHC (Figures 5A, 5B).

The complementary DNAs coding for chains α and β of murine class II molecules of IAb haplotype and the invariant murine chain were inserted in the NT eukaryote expression vectors in which cDNA transcription is under the control of an alpha SR promoter. Each of the plasmids also carries a resistance gene to hygromycine (for the α IAb chain), to neomycine (for the β IAb chain) or zeocine (for the invariant chain). After transfection of the RBL2H3 cells by electroporation (material and methods), the cells were selected on the basis of their resistance to the three antibiotics, then the resistant cells were cloned by limit dilution and characterised for

expression of the IAb molecules by flow cytometry using the specific Y3P antibody.

Figure 5 shows some of the results obtained. Flow cytometry analysis shows that the RBL IAbIi cell is recognised by the Y3P antibody (specific to IAb molecules) in equivalent manner to the B-lymphocyte line B 414, whereas no labelling was detected on the initial RBL line (Figure 5A). Since morphologic analysis by microscopy established that the molecules of murine class II IAb are, like the human DR1 molecules, accumulated in the secretion granules of the cell (not shown), this led us to searching their localisation in the exosomes. Exocytosis of the cells was initiated by the addition of 1 mM ionomycine, and the exosomes obtained were purified by differential ultracentrifugation (cf example 1.2). Western blotting of these exosome preparations shows that they contain class II MHC murine molecules identical to those detected in a control cell lysate (Figure 5B).

These results demonstrate that class II human molecules (DR1) as well as murine molecules (IAb) may be expressed and can accumulate in the exosomes of the corresponding RBL 2H3 lines.

5 - <u>Morphological characterisation of the exosomes produced by RBL 2H3 (Figure 6)</u>

Observations under confocal microscopy on frozen immunolabelled slices of RBL cells reveal the presence of numerous intracellular compartments filled with membranes. The most part of these membranes

correspond to vesicles which fill the lumen of the compartments (Figure 6A). When transfected in these cells the class II molecules accumulate in the compartments having internal vesicles and are visualised in particular in association with the membrane of these vesicles (Figure 6A).

When the RBL cells are stimulated such as to induce their degranulation (IgE-Antigen or ionomycine), the intracellular compartments fuse with the plasma membrane. The internal vesicles with which the class II molecules are associated are released into the extracellular medium. These vesicles are then called exosomes.

The method of choice in electronic microscopy to examine the morphology of exosomes and their protein content is the "whole mount" method. With this technique it is possible to visualise whole exosomes free of any other cell content. With this method it is also possible to detect, with great efficacy, molecules associated with the membrane of the exosomes. By using this technique we observed that the exosomes secreted by the RBL cells are of heterogeneous size from 30 to 120 nm and have variable density to electrons (Figure 6B). The class II molecules are abundant in the population of vesicles having a size of 80 to 100 nm and having average density to electrons. The vast majority of these vesicles enriched with class II molecules are dish-shaped (Figure 6C).

6- Immobilisation of exosomes on support media (Figure 5C).

This example describes the fixation of exosomes on solid supports and shows that the exosomes fixed in this way maintain their functional properties. These new products (exosome supports) may be used to characterise and analyse exosomes ; or as diagnostic or reagent products to detect and/or stimulate T-lymphocytes *in vitro* for example.

Different preparations of exosomes produced by RBL cells whether or not expressing class II human or murine molecules were incubated with 4 micron latex beads activated by aldehyde sulfate. More particularly, the exosomes purified from degranulation supernatants of RBL 2H3 were washed in PBS (centrifuging at 50000 rpm on TLA 100.4 for 30 minutes). 30 µg exosomes are mixed with 10 µl of latex beads collected sterilely, homogenised then incubated for 10 min to 15 min at room temperature. The bead volume is then completed up to 1 ml with 1x PBS then incubated at room temperature for 2h. Subsequently, the beads crosslinked with exosomes are:

- saturated by adding final 100 mM glycine (30 min at room temperature),
- centrifuged at 2200 g for 2 min at 4°C, then the bead residue is collected in 1 ml 1x PBS 3%SVF 0.01%NaN3.

To use the beads for cytofluorometry :

- wash the bead residue two to three times in 1xPBS 3%SVF 0.01%NaN3,

- collect in 1mL 1xPBS 0.01%NaN3,

- use between 5 µL and 20 µL per point and incubate conventionally the second antibody in the first well. Reading is made on Facscalibur (Becton Dickinson).

With this technique it was possible to cover the surface of these latex beads with exosomes while preserving their structure.

When the exosomes are on the beads, their handling is made easier. They can for example be centrifuged at low speed and detected by conventional flow cytometry techniques. Figure 5C shows a few examples of detection, by flow cytometry, of different proteins entering into the composition of exosomes. Latex beads activated with aldehyde sulfate were incubated either with exosomes produced by the non-transfected RBL 2H3 cells, or with exosomes of RBL 2H3 cells expressing the class II human molecules DR1 and the IiHA construct or the class II murine molecules IAb, or with fetal calf serum (FCS) as a control. The latex beads prepared in this way were then incubated with different monoclonal antibodies ; AD1 recognising the CD63 molecule of the rat present in the secretion granules of RBL 2H3, Y3P antibody specific to molecules IAb, and the L243 antibody specific to DR1 molecules. These different antibodies were exposed by secondary

antibodies coupled to phycoerythrine, then the labellings obtained were analysed by flow cytometry with a FACScalibur (Beckman).

It is thus observed that the CD63 molecule is evidently present on all the exosomes derived from cell RBL, whether or not expressing class II molecules, whereas latex beads coated with IAb exosomes are specifically recognised by Y3P and not by L243, whereas contrariwise, the DRIiHA exosomes are recognised by L243 and not by Y3P. None of these antibodies recognises latex beads covered with fetal calf serum (Figure 5C).

These results show that this technique enables the sensitive, specific detection of the expression of different proteins entering into the composition of exosomes. Example 8 also shows that by means of such products (exosome supports) it is possible also to detect or stimulate the proliferation of specific T-lymphocytes.

7- <u>Manipulation of the composition of the exosomes (Figure 7)</u>

Exosomes are vesicles bounded by a lipid bi-layer in which are inserted a great number of molecules such as the class II MHC molecules or CD63 previously mentioned. Inside these vesicles are found the cytoplasmic regions of the preceding transmembrane molecules, but also soluble proteins derived from the cell cytosol. To demonstrate that it is possible to

modify at will the content of these vesicles, we used Green Fluorescent Protein (GFP) as tracer.

The cDNA coding for GFP was fused at the COOH terminal end of the beta chain of the human DR1 molecule. This construct, inserted in the NT expression vectors carrying the resistance gene to hygromycine, was co-transfected in cell RBL 2H3 with a vector carrying the alpha chain of DR1 and the resistance gene to neomycin. Cells resistant to these two antibiotics were selected then sorted positively for the expression of GFP.

More particularly, we made a construct binding the cytoplasmic part of the DRβ chain (at C-ter) to the N-ter end of GFP. The cDNA of DRβ has a PstI site at position 565. A fragment of approximately 200 base pairs of the 3′ side of this cDNA was amplified by PCR from the vector pcDNA3/RSV/Dra by means of 2 oligonucleotides including the Pstl site for 5′ and the NcoI site for 3′ which in addition eliminated the stop codon. The PCR fragment obtained was digested with PstI and NcoI and cloned in the same sites of the vector pEGFP N1 (Clontech). The resulting plasmid, digested with PstI and Xbal enabled the release of a fragment corresponding to the last 30 amino acids of DRa followed by GFP. In parallel, the plasmid pcDNA3/RSV was digested with EcoRV/PstI, thereby releasing a fragment corresponding to the remainder of the DRa chain (from the start up as far as site PstI).

The two fragments were then assembled and cloned in pcDNA3/CMV between EcoRV and Xbal.

Analysis of these cells (DR1 GFP) by flow cytometry shows that the cells recognised by the L243 antibody, specific to the DR1 molecules, also emit green fluorescence detected in canal FL1, while cells transfected with the alpha and beta chains of DR1 not comprising GFP do not emit any fluorescence in canal FL1 (Figure 7A).

In order to demonstrate that GFP is truly contained in the exosomes of cell RBL 2H3, exosomes derived from cells RBL DR1 GFP were prepared by differential ultracentrifugation then analysed by Western blot (Figure 7B) and flow cytometry after crosslinking on latex beads (Figure 7C). Under Western blotting, a specific antibody of GFP detects, in cell DR1 GFP and in its exosomes a protein of 65 kDa corresponding to the molecular weight of the beta chain of DR1 fused with GFP (Figure 7B) whereas no signal is detected in the cell lysates of cell RBL2H3 whether or not expressing the DR1 molecule alone. Comparison between latex beads crosslinked with fetal calf serum or with exosomes derived from the DR1 GFP cell, shows that solely the beads coated with exosomes induce fluorescence in canal FL1 (FITC) and are recognised by the L243 antibody, specific to DR1, detected by a secondary antibody coupled to phycoerythrine.

These results therefore demonstrate that it is possible to insert an exogenous protein inside exosomes

produced by the RBL 2H cell. These results further show that it is possible to direct a protein into an exosome by expression in the producer cell, in the form of fusion with a transmembrane molecule such as a molecule of the MHC.

8 - Functional characterisation of exosomes (Figure 8)

This example shows that the exosomes produced by the RBL 2H3 cell, carrying class II molecules of the MHC, are able to bind an antigenic peptide and to stimulate a T-lymphocyte expressing a specific receptor of this peptide-MHC class II complex.

Exosomes produced from RBL DR1 IiHA cells labelled with Green Cell Tracker were incubated in the presence of two types of T cells : THAs which have a TCR specific to HLA-DR1/HA complexes, and wild T Jurkats free of said receptor. Binding experiments are conducted using a 96-well multidish with round-bottomed wells, in RPMI 1640 medium supplemented with 10% fetal calf serum, buffered with 10mM Hépès up to a final volume of 50µl per well, 10^5 T cells per well and variable quantities of fluorescent exosomes for 3 hours at 37°C. Then two washings are made in the same medium before analysing the cells collected with the FACS in 400 µl PBS.

A dose effect range was drawn up showing that the labelling intensity of the THAs was proportional to the quantity of exosomes used. 10^8 RBL DR1 IiHA cells

gave 700 µl exosomes. Doses ranging from 32 to 2 µl exosomes per well were tested. The labelling obtained with 16 µl per point was retained, which corresponds to the production of exosomes made by 2.3 million cells (results not shown).

The results obtained show that the THA population is labelled by the fluorescent exosomes which yields detectable fluorescence at FL1 by cytofluorimetry, while the wild Jurkats are not modified (Figure 8A).

The same type of labelling was conducted, but after binding of the exosomes, the T cells were incubated with an AD1 mouse monoclonal antibody antirat CD63, subsequently developed by donkey antibody antimouse IgG labelled with phycoerythrine (Jackson Immuno Research, Wst Grove, PA). The same labelling was made in parallel on T cells that had not been exposed to exosomes. It is seen (Figure 8B) that only exosome-binding THA cells, as proved by their fluorescence at FL1, are also labelled at FL2 which indicates the new presence of rat CD63 on their surface.

Our results consequently show that the fluorescent exosomes can be used to visualise by flow cytometry populations of T-lymphocytes that are specific to HLA-DR/antigenic peptide complexes carried by the exosomes.

To assess the capacity of the exosomes to stimulate a T-lymphocyte in a manner dependent upon on

the presence of a peptide, the exosomes obtained from the DR1 GFP cell were crosslinked on latex beads then incubated in the presence of cells of the T Jurkat lymphocyte line whether or not expressing a specific T receptor of the complex DR1-HA peptide 307-319.

The latex beads were prepared in the same way as for flow cytometry but were washed in complete medium (RPMI, 10% fetal calf serum, 1% Penicillin-Streptomycine-Glutamine, 0.1% β-mercaptoethanol, 4% sodium pyruvate). For the stimulation of the T-lymphocytes, each bead residue was collected in 100 µL : 50 µL, deposited in the first well of a 96-well multidish and 50 µL diluted in halves. A control was conducted proceeding in the same manner with beads incubated in fetal calf serum. The T cells (T Jurkat Pasteur and THA1.7) were adjusted to 10^6 cells/mL and 50 µL were deposited per well. The peptide diluted to 15 µM in complete medium was also added to the proportion of 50 µL per well. In the peptide-free series, 50 µL of complete medium were added per well. The culture dish was placed in an incubator (37°C, 5% CO2, 2O) for 20 hours then the supernatant was collected and the concentration of IL2 was evaluated with a CTL.L2 test.

It is observed that the addition of the HA petide (5mM) specifically induces stimulation of the T Jurkat cell expressing the receptor of the DR1-HA (THA) complex, whereas it has no effect on the control T-lymphocyte (T Jurkat). Also, some exosomes are

incapable of stimulating THA in the absence of HA peptide (Figure 8C).

9 Exosomes of the human masctocyte line (Figure 9)

This example shows that exosomes modified according to the invention may be produced from other cells, especially human cells. In particular, the results which we obtained demonstrate that the mastocyte line of human MHCI origin is able to produce exosomes under the impulse of an increase in intracellular calcium and under identical conditions to those which induce the secretion of exosomes by the rat line RBL 2H3.

Characterisation of the MHCI line by flow cytometry indicates that the surface of these cells expresses class I molecules of the MHC (W6.32) but not class II molecules (L243). Also, they are positive for molecules CD9, CD63 and CD81, but negative for molecules Lamp1 and Lamp 2 (Figure 9A).

Exosomes were produced from the MHCI line through the addition of ionomycine (1mM) but purified from supernatants by differential ultra-centrifugation. Their composition was analysed by flow cytometry after crosslinking on latex beads and by Western blot.

Analysis of the exosomes with the method using latex beads shows that they carry molecules Lamp1, CD9, CD63, CD81 and class I molecules of the MHC but not class II molecules nor the Lamp2 molecule (Figure 9B).

In addition, the morphology observed under electronic microscopy of these exosomes is identical to those produced by the RBL 2H3 line. Finally, the protein composition observed by Western blot confirms these results since with this technique we found molecules CD63, Lamp1, MHC class I, whereas Lamp2, MHC class II remained negative (Figure 9C).

To conclude, line MHCI appears to be the human homologue of the RBL-2H3 rat line through its capacity, after an increase in intracellular calcium, to produce exosomes which have the same structural and molecular characteristics. With these cells it is therefore possible to produce recombinant human exosomes expressing class II molecules of the MHC or any other molecule specifically addressed to them .